A promoter derived from phytosulfokine precursor gene

BACKGROUND OF THE INVENTION

1. Field of the invention

[0001] This invention relates to a promoter of phytosulfokine precursor derived from rice and a transgenic plant produced by incorporation of the promoter to activate expression of an exogenous structural gene.

2. Description of Related Art

[0002] In the field of plant genetic engineering, a promoter is ligated to upstream of an exogenous gene of target, which is a conventional technique for over-expression of the exogenous gene. In many cases, expression of the exogenous gene is not sufficient without existence of such promoter. Various promoters are utilized for this purpose and the method using cauliflower mozaic virus is the most conventional technique in this art. Here, a promoter means a regulatory region existing 5'-upstream of a structural gene. It is known that binding of RNA polymerase to a promoter serves as an initiation signal of transcription.

[0003] The method utilizing cauliflower mozaic virus (CAMV) *35S* promoter is an excellent method for over-expression of an exogenous gene. However, in some cases, the extent of expression of the exogenous gene is not sufficient, depending on the exogenous gene to be incorporated and the species of the host plant which is the target of gene incorporation. Thus, there have been strong demands on a promoter with higher activity. It is the object of this invention to obtain a novel promoter exhibiting higher potency to activate a structural gene, compared with the conventional CAMV*35S* promoter.

[0004] The inventors noticed phytosulfokine (PSK), which is a peptide growth factor of a plant, and performed investigation on the growth factor. PSK is one of growth factors contained in so-called "conditioned medium:CM", a

medium once used for cell culture. It is known that PSK is secreted into extra-cellular medium and functions in the manner like autocrine. At performance of plant cell culture, various known hormones or nutritional elements are added to the culture medium as a conventional technique. However, in some plant species, cell culture is itself difficult or rate of cell proliferation is extremely slow. Moreover, when the density of a plant cell is lower than necessary, proliferation of the plant cell becomes difficult. Even in such cases, PSK is effective to enhance proliferation of a plant cell. It is also known that, the structure of PSK-α and PSK-β are defined by the following sequences wherein tyrosine residues of the PSKs are sulfated by post-translational modification.

PSK-α : Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln

PSK-β : Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr

[0005] The PSK-α and PSK-β are extra-cellular secreted peptides bio-synthesized in the form of their precursor, sulfated and processed during their transition via trans-Golgi network. The cDNA sequence of *Oryza sativa* phytosulfokine (*OsPSK*) have been already determined, using the technique of cDNA cloning. Moreover, the cDNA thus obtained and the polypeptide encoded by the cDNA are described in Japanese Patent Publication No. 11-079612. Incorporation of said gene into a plant would enhance proliferation of a plant.

SUMMARY OF THE INVENTION

[0006] One aspect of this invention is a promoter consisting of a base sequence of following (a), (b) or (c):

(a) a base sequence represented by base numbers -3359 to -1 shown in SEQ:ID NO:1 in the sequence list,

(b) a base sequence in which a part of said base sequence (a) is deleted or another base sequence is added to said base sequence (a) or a part of base sequence (a) is substituted with another base sequence, the base sequence (b) exhibiting activity to enhance expression of a structural gene existing downstream of the promoter,

or

(c) a base sequence that hybridizes with the base sequence (a) under stringent conditions.

[0007] Further aspect of this invention is a promoter consisting of a base sequence of following (d), (e) or (f):

(d) a base sequence represented by base numbers -1911 to -1 shown in SEQ:ID NO:2 in the sequence list,

(e) a base sequence in which a part of said base sequence (d) is deleted or another base sequence is added to said base sequence (d) or a part of base sequence (d) is substituted with another base sequence, the base sequence (e) exhibiting activity to enhance expression of a structural gene existing downstream of the promoter,

or

(f) a base sequence that hybridizes with the base sequence (d) under stringent conditions.

[0008] Further aspect of this invention is a promoter consisting of a base sequence of following (g), (h) or (i):

(g) a base sequence represented by base numbers -1034 to -1 shown in SEQ:ID NO:3 in the sequence list,

(h) a base sequence in which a part of said base sequence (g) is deleted or another base sequence is added to said base sequence (g) or a part of base sequence (g) is substituted with another base sequence, the base sequence (h) exhibiting activity to enhance expression of a structural gene existing downstream of the promoter,

or

(i) a base sequence that hybridizes with the base sequence (g) under stringent conditions.

[0009] Further aspect of this invention is a promoter consisting of a base sequence of following (j), (k) or (l):

(j) a base sequence represented by base numbers -563 to -1 shown in SEQ:ID

NO:4 in the sequence list,

(k) a base sequence in which a part of said base sequence (j) is deleted or another base sequence is added to said base sequence (j) or a part of base sequence (j) is substituted with another base sequence, the base sequence (k) exhibiting activity to enhance expression of a structural gene existing downstream of the promoter, or

(l) a base sequence that hybridizes with the base sequence (e) under stringent conditions.

[0010] Further aspect of this invention is a gene encoding phytosulfokine precursor consisting of a base sequence represented by base numbers -3359 to 2033 shown in SEQ:ID NO:5 in the sequence list.

[0011] Further aspect of this invention is a plasmid in which above described promoter was incorporated. Moreover, a transgenic plant cell in which above described promoter was incorporated to activate expression of a structural gene existing downstream of the promoter is also within the range of this invention. Moreover, a transgenic plant body in which above described promoter was incorporated to activate expression of a structural gene existing downstream of the promoter is also within the range of this invention.

[0012] Further aspect of this invention is a method to activate expression of an endogenous structural gene or an exogenous structural gene in a plant by incorporation of above described promoter into upstream of the structural gene.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The above and other objects and features of the present invention will be further explained in detail hereinafter from consideration of the following description taken in connection with the accompanying drawings, in which:

Fig. 1 is a photograph of blotting analysis performed on genomic DNA of rice Oc cell using cDNA of *OsPSK* as a probe;

Fig. 2 is a schematic figure showing restriction map of *OsPSK*

genomic gene and genomic structure of the *OsPSK* gene;

Fig. 3 is a photograph showing result of primer extension reaction and S1 mapping;

Fig. 4 is a figure showing base sequence of *OsPSK* gene and its deduced amino acid sequence;

Fig. 5 is a figure showing structure of *OsPSK*-intron-GUS constructs;

Fig. 6 is a graph showing GUS activity of transformed rice Oc cells transformed with *OsPSK*-intron-GUS constructs;

Fig. 7 is a photograph showing regulation of *OsPSK*::GUS gene by auxin and cytokinin.

DETAILED DESCRIPTION OF THE INVENTION

[0014] The inventor noticed the above-described knowledge and investigated to obtain a promoter that regulates expression of the *OsPSK* gene. Meanwhile, the base sequence described in Japanese Patent Publication No. 11-079612 is a cDNA sequence obtained by transcriptase reaction using mRNA as a template. Therefore, the sequence does not include non-translated region of the gene and the region corresponding to intron. In general, a regulatory region like a promoter is not translated. Therefore, it is requisite to prepare genomic DNA library, originated from total DNA of a cell, to obtain genomic DNA of *OsPSK* including regulatory region. Then, the inventors have prepared genomic library of *OsPSK* and performed cloning by plaque hybridization using the cDNA as a probe. As the result, full-length genomic sequence of PSK precursor gene was obtained and non-translated region and intron were included in the sequence. In the genomic sequence, it was revealed that a promoter exists upstream of its open reading frame region and the promoter includes various consensus sequences. Beta-glucuronidase (GUS) was incorporated downstream of the promoter and the potency of the promoter to activate expression of GUS gene was investigated. As the result, the potency of this promoter to activate GUS

gene was higher than that of CAMV*35S* promoter. Therefore, it was shown that the promoter might be a useful tool that enables constitutive activation of expression of an exogenous gene.

[0015] This invention is a gene encoding phytosulfokine precursor polypeptide, consisting of a base sequence defined by base numbers from -3359 to 2033 in SEQ.ID No.5 in the sequence list. This is a genomic DNA sequence encoding PSK precursor, obtained by screening of the genomic library by plaque hybridization using 32P-labeled cDNA as a probe. The genomic DNA sequence and the cDNA sequence of the PSK precursor, which have already obtained, were compared and it was revealed that the genomic DNA sequence consisted of two exons and one large intron. The sequence encoding PSK, consisting of 5 amino acids, existed in the second exon. In SEQ.ID No.5 in the sequence list, base numbers from 1 to 1858 corresponds to the transcriptional region. The region of base numbers from 246 to 1395 corresponds to the intron and the region of base numbers from 1396 to 1858 corresponds to the second exon including PSK coding region. Moreover, the region of base numbers from 1396 to 1521 corresponds to the 3' downstream purlieu sequence. A putative TATA box was found at the position of –68 and consensus sequences of potential regulatory elements were found further upstream of the sequence. Those are, one CAAT-box, three CCAAT-boxes, three SSREs (shear-stress-responsive element), one enhancer core-like sequence and three E-boxes. Those are consensus sequences commonly recognized among many organisms and involved in regulation of transcription. It is known that a certain protein binds to each of these consensus sequences. When such binding occurs, the frequency of transcription is regulated by interaction between the protein and the DNA.

[0016] As described in the following embodiment, a plasmid containing fusion gene comprising 5'-region of *OsPSK* gene and β-glucuronidase (GUS) gene was prepared. The effect of 5'-region of *OsPSK* gene on GUS activity

was investigated. As the result, the region containing 5'-regulatory elements enhanced activity of GUS gene and the potency was higher than that of CAMV*35S* promoter. Such promoter sequence derived from 5' region of *OsPSK* gene is defined by base numbers from –3359 to –1 in SEQ.ID No.1 in the sequence list. The promoter is regulatory region of *OsPSK* gene and the region corresponds to the sequence utilized to incorporate into plasmid pIG121-6 in the following example (refer to Fig 5). In the *OsPSK* gene, the sequence is the longest sequence of the promoter that exhibits activity as a promoter and if a sequence longer than it was adopted, the activity as a promoter would decrease significantly.

[0017] The 5' region of *OsPSK* gene maintains activity as a promoter, even if a shorter sequence of SEQ.ID No.1 is adopted. Promoter consisting of the sequence defined by base numbers from –1911 to –1 in SEQ.ID No.2 in the sequence list is one of such promoter. The region corresponds to the sequence was utilized to incorporate into plasmid pIG121-4 in the following example (refer to Fig 5). In the *OsPSK* gene, this sequence corresponds to the region that exhibits maximum activity as a promoter.

[0018] The promoter consisting of the sequence defined by base numbers from –1034 to –1 in SEQ.ID No.3 in the sequence list, is also one of such region of the promoter that maintains activity as a promoter. The region derived from the *OsPSK* gene corresponds to the sequence utilized to incorporate into plasmid pIG121-3 in the following example (refer to Fig 5).

[0019] Furthermore, the promoter consisting of the sequence defined by base numbers from –563 to –1 in SEQ.ID No.4 in the sequence list, is also one of such region of the promoter that maintains activity as a promoter. The region derived from the *OsPSK* gene corresponds to the sequence utilized to incorporate into plasmid pIG121-2 in the following example (refer to Fig 5). This sequence corresponds to the minimum region of *OsPSK* gene that exhibits activity as a

promoter. If a sequence shorter than it was adopted, the activity as a promoter would decrease significantly.

[0020] According to technique of gene recombination, artificial modification can be achieved at a specific site of basic DNA, without alteration or with improvement of basic characteristic of said DNA. Concerning a gene having native sequence provided according to this invention or modified sequence different from said native sequence, it is also possible to perform artificial modification such as insertion, deletion or substitution to obtain gene of equivalent or improved characteristic compared with said native gene. Moreover, a gene with such mutation is also included in the range of this invention. A promoter consisting of a base sequence in which a part of said promoter consists of base sequence shown in SEQ ID NO: 1 is deleted, substituted or added with one or more bases means a promoter in which a part of said promoter consists of base sequence shown in SEQ ID NO: 1 is deleted, substituted or added with one or more bases while maintaining activity as a promoter to enhance expression of a structural gene existing in the downstream of the promoter. Such promoter exhibits homology 70% or more, preferably 80% or more and still preferably 90% or more with the amino acid sequence shown in SEQ ID NO: 1 in the sequence list. Moreover, concerning such promoter, the number of bases deleted, substituted or added compared with the base sequence shown in SEQ ID NO: 1 is 20 or less, preferably ten or less, and more preferably five or less. In addition, such promoter hybridizes with the base sequence shown in the SEQ ID NO: 1 in the sequence list thereof under stringent condition. Then, a promoter consisting of a base sequence in which a part of said promoter consists of base sequence shown in SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 4 is deleted, substituted or added with one or more bases means the same variations of the base sequences.

[0021] A plasmid, in which the promoter derived from *OsPSK* gene was

incorporated, is also within the scope of this invention. In the following example, pIG121 plasmid containing CaMV*35S* promoter and GUS reporter gene was adopted, but the scope of this invention is not to be limited to it. For example, other plasmids conventionally utilized in the art, such as pIG122, pBI101, pBI121, pBI221, pAct-nos/Hmz, pMAT037, pTA7001 and pTA7002, can be also adopted.

[0022] A transformed plant produced by incorporation of the promoter of this invention to enhance expression of an exogenous gene is also within the scope of this invention. The example of plants, preferred as a target of incorporation of the promoter of this invention to activate expression of an exogenous gene, may include monocotyledonous plants, such as rice, lily, maize, asparagus and wheat, as well as dicotyledonous plants, such as tobacco, *Arabidopsis thaliana*, carrot, soybean, tomato and potato. In principal, any plant can be adopted to incorporate the promoter of this invention thereby activate expression of an exogenous gene. In the following example, *Agrobacterium tumefaciens* LBA4404 strain was adopted, but not the scope of this invention is not to be limited to the strain. According to the species of the plant, which is the target of incorporation, an Agrobacterium strain conventionally utilized in this art can be properly selected and adopted.

[0023] In principal, any useful structural gene can be adopted as an exogenous gene of target to be incorporated downstream of the promoter to activate expression of the exogenous gene. In the following embodiment, GUS gene was incorporated but the scope of this invention is not to be limited to it. The example of exogenous genes to be incorporated may include a gene encoding a factor that enhances growth of a plant, a gene involved in resistance against various environmental stresses, a gene involved in resistance against disease injury of a plant, a herbicide resistance gene and a gene encoding an enzyme involved in synthesis of a useful secondary metabolite.

[0024] Moreover, a plant body obtained from a transgenic plant cell thus produced to activate expression of an exogenous gene and a method to produce a transformed plant cell are also within the scope of this invention. In the following embodiment, this invention is elucidated in detail using rice culture cell as an example, above description and following embodiment is not to be considered to limit the scope of this invention.

EMBODIMENT

(Plant cell culture)

[0025] Rice Oc culture cells (Baba *et al.*, 1986) were subcultured at 25±2°C in the dark at 120 rpm in fresh Murashige and Skoog medium (MS; Murashige and Skoog, 1962) supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) at regular intervals of 2 weeks.

(Genomic DNA extraction and Southern blot analysis)

[0026] Genomic DNA was extracted from rice Oc culture cells cultured for 14 days using the CTAB method (Murry and Thompson, 1980), digested with restriction endonucleases, separated by electrophoresis on 0.8% (w/v) agarose gels, and blotted onto Biodyne nylon membranes (Pall, Port Washington, NY, USA) in alkaline transfer buffer (0.4 N NaOH/0.6 N NaCl). Southern hybridization was performed in a solution of 5 x SSC, 0.5% SDS, 5 × Denhardt's solution and 500 μg/ml Salmon sperm DNA at 50°C or 65°C using the *OsPSK* cDNA ^{32}P-labeled with a Random Primed DNA Labeling Kit (Takara, Tokyo, Japan). After hybridization, washing was performed with 2 × SSC at 25°C 15 min for 3 times and then 2 × SSC containing 0.1% SDS at 50°C or 65°C 15 min for 3 times.

(Construction and screening of a genomic library)

[0027] Genomic DNA (50 μg) was partially restricted with *Sau*3A I to generate *Bam*HI-compatible fragments ranging in size from 9 to 23 kb. The genomic fragments (0.3 μg) were inserted into the *Bam*H I site of EMBL3

vectors that had been digested with *Bam*HI and *Eco*RI (Stratagene, La Jolla, CA, USA) and packaged with Gigapack III Gold Packaging Extract (Stratagene) to construct a genomic library. This was screened by plaque hybridization with the ^{32}P-labeled *OsPSK* cDNA. Hybridization and washing were executed under high stringency conditions at 65°C as described above.

(Subcloning and DNA sequencing)

[0028] Restriction mapping of positive genomic clones was carried out by single and double restriction enzyme digestion. A series of fragments spanning the *OsPSK* gene were excised from positive phages, gel-purified, and subcloned into the corresponding sites of pBluescript II-KS (pBS) plasmids. Deletion clones were generated for DNA sequencing with a Kilo Sequencing Kit (Takara) according to the protocol recommended by the manufacturer. The plasmids containing the deleted fragments were introduced into *Escherichia coli* strain JM109 and sequenced completely on both strands. Double-stranded DNA sequencing reactions were run using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA), and analysis of the DNA sequence data was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) in accordance with the manufacturer's protocols.

(Determination of transcriptional start site)

[0029] The transcriptional start site was established by both primer extension and S1 nuclease analysis. Total RNA extracted from 2-week-old Oc culture cells after transplanting and a 32 nucleotide long primer with the sequence 5'-AGCAGGAGGAGAGCAAGGCATAGGAGGCAGAG-3' (SEQ ID NO: 6) which is complementary to a region 32 nucleotides downstream of the ATG start codon in the *OsPSK* cDNA were used for both analyses. The oligonucleotide primer was 5'-end-labeled with 10 U of T4 polynucleotide kinase (Takara) and 30 μCi of [γ-^{32}P]-ATP (3000 μCi/mmol). End-labeled primer was annealed with 10 μg of total RNA for 1 h at 60°C. The primer extension reaction was carried

out according to the published procedure (Sambrook *et al.*, 1989) at 37°C for 1 h with 20 U of Moloney murine leukemia virus reverse transcriptase (Stratagene). For S1 mapping, a 3.6-kb EcoRI fragment from the *OsPSK* gene and the end-labeled primer were used to synthesize one strand DNA. A 315-b fragment was excised and purified from the synthesized DNA with PstI and used as the S1 probe, hybridized to 30 μg of total RNA at 60°C and incubated with 300 units/ml S1 nuclease (Takara) for 30 min at 30°C. The reaction products in both experiments were run side by side on a 6% polyacrylamide gel containing 7 M urea.

(Construction of chimeric genes)

[0030] The plasmid pIG121 contains the cauliflower mosaic virus (CaMV) *35S* promoter, a modified intron of the castor bean catalase gene, and the GUS reporter gene (Akama *et al.*, 1992). This CaMV *35S*- Intron-GUS reporter gene is expressed in plants but not in cells of *Agrobacterium tumefaciens* (*A. tumefaciens*) (Ohta *et al.*, 1990). The pIG121 harbors a kanamycin resistance gene which can be used to select transformants. The plasmid pIG121 was digested by *Hin*dIII-*Xba*I to remove the CaMV *35S* promoter and various fragments from the 5' region of the *OsPSK* gene were inserted into the *Hin*dIII-*Xba*I sites of the binary vector to construct chimeric genes as follows. A 241-bp *Pst*I-*Bgl*II fragment from the 5' region of the *OsPSK* gene was subcloned into pBS at the *Pst*I-*Bam*HI sites to generate pBS-1. The fragment was excised by *Hin*dIII-*Xba*I from pBS-1 and cloned between the same sites of the promoter-less pIG121 to construct pIG121-1. Introduction of a 662-bp *Hind*III-*Xba*I fragment from pBS-2 containing the *EcoRV-Bgl*II fragment into the same sites of the promoter-less pIG121 produced pIG121-2. The 1.1-kb *Hin*dIII-*Bgl*II fragment of the 5' region of the *OsPSK* gene was subcloned into the *Hin*dIII-*Bam*HI sites of pBS to produce pBS-3. The plasmid pIG121-3 was generated by introduction of the 1.1-kb fragment from pBS-3 into the *Hin*dIII-*Xba*I sites of the promoter-less pIG121. The 2-kb *Bgl*II-*Bgl*II fragment of the 5' region of the

OsPSK gene was subcloned into the *Bam*HI site of pBS to produce pBS-4. A 877-bp *Hin*dIII-*Hin*dIII fragment was excised from pBS-4 and inserted into the same site of pIG121-3 in both possible orientations to produce pIG121-4 and pIG121-5. The 3.7-kb *Eco*RI-*Eco*RI fragment of *OsPSK* was isolated and cloned into the same site of pBS to produce pBS-5, which was then cleavaged by *Hin*dIII and the 2.3-kb fragment inserted into the same sites of pIG121-3 in both possible orientations to construct pIG121-6 and pIG121-7.

[0031] Here, the sequence incorporated into pIG121-1 corresponds to the region represented by base numbers from –148 to –1 in SEQ ID NO: 1 in the sequence list. The sequence incorporated into pIG121-2 corresponds to the region represented by base numbers from –563 to –1. The sequence incorporated into pIG121-3 corresponds to the region represented by base numbers from –1034 to –1. The sequence incorporated into pIG121-4 corresponds to the region represented by base numbers from –1911 to –1. The sequence incorporated into pIG121-5 is the same region incorporated into pIG121-4, but the region corresponding to base numbers from –1911 to -1034 is incorporated in the reverse orientation. The sequence incorporated into pIG121-6 corresponds to the region represented by base numbers from –3359 to –1. The sequence incorporated into pIG121-7 is the same region incorporated into pIG121-6, but the region corresponding to base numbers from –3359 to -1034 is incorporated in the reverse orientation.

(*Agrobacterium*-mediated transformation of rice Oc cells)

[0032] The constructs were transformed into A. *tumefaciens* strain LBA4404 by triparental matting. For *Agrobacterium*-mediated transformation, *A. tumefaciens* cells were grown for 3 days on AB agar medium containing 50 mg/liter kanamycin at 30°C in the darkness. The bacteria were collected with a small spoon, and suspended in AAM medium (Yang *et al.*, 2000a) at a density of OD_{600} = 0.2. Oc cells (0.5 ml packed cell volume) that were pre-cultured for

3 days at 25°C in the dark on fresh MS medium supplemented with 1.0 mg/l of 2,4-D prior to infection were immersed in 4 ml of the bacterial suspension in 90 mm × 20 mm Petri plates (Terumo, Tokyo, Japan). The plates were sealed with parafilm and co-cultivation was carried out in the dark at 28°C for 3 days. After 3 days of inoculation, cultures were collected into Falcon 2097 tubes. Oc cells were collected by centrifugation at 1,000 rpm (MX-160 centrifuge, TMA-27 angle rotor; Tomy) for 1 min, resuspended in MS medium, mixed well by gentle vortexing, and then centrifuged at 1,000 rpm for 1 min. After centrifugation, the supernatant was discarded. This rinsing process was repeated for 3 to 5 times to exclude *A. tumefaciens* cells. The rinsed Oc cells were cultured on fresh MS medium supplemented with 1.0 mg/l of 2,4-D, 50 mg/l kanamycin, and 250 mg/l cefotaxime to select transformed cells.

(Quantitative analysis of GUS activity)

[0033] More than 5 independent transformed cell lines for each construct were cultured for 7 days on selection medium for measurement of GUS activity. Total soluble protein was isolated from the transformed Oc cells in a GUS extraction buffer (Jefferson *et al.*, 1987) and activity was quantitatively assayed by the fluorometric reaction procedure of Jefferson *et al.* (1987) using 4-methylumbelliferyl-β-D-glucuronide (Sigma, St. Liouis, MO, USA). The amount of protein was determined with a Bio-Rad Laboratories kit by the method of Bradford (1976).

(RNA isolation and Northern blot analysis)

[0034] Oc cells transformed with the plasmid pIG121-4 were cultured for 0, 12, 24, 48, and 72 h, respectively, in MS media supplemented with different combinations of phytohormones: 2 mg/l of 2,4-D only, 2 mg/l of 6-BA only, or 1 mg/l each of 2,4-D and 6-BA. Total RNA (20 μg per lane) isolated from various samples by a published method (Chomczynski, 1993) was denatured and fractionated by electrophoresis on 1.2% (w/v) agarose gels containing 2.2 M

formaldehyde. The RNAs were subsequently transferred to Biodyne nylon membranes (Pall) in 20 × SSC. The filters were hybridized to a random-primed probe of the gusA gene as described above.

(*Copy* number of *OsPSK* gene)

[0035] The inventors previously characterized *OsPSK* cDNA in rice encoding the precursor of PSK-α, a peptide growth factor identified from plants. Prior to the isolation of genomic clones corresponding to *OsPSK* cDNA, the copy number of this gene in the rice genome was investigated by genetic Southern blotting. DNA blot analysis under low stringency conditions using the full-length *OsPSK* cDNA as a probe revealed only one band in DNA digested with three different restriction enzymes, except *Eco*RI that generated two strong and one weak hybridizing bands (Yang *et al.*, 1999). When the Southern blot was hybridized to the probe under high stringency conditions, only the strong bands were detected in *Eco*RI-digested DNA (data not shown). These two hybridizing bands were expected by the restriction site residing in the coding region of *OsPSK* cDNA. To verify this notion, the inventors reprobed the blot using a 300-bp fragment from the 5' terminus of *OsPSK* cDNA. As expected, only one band of 3.6 kb was hybridized under either low or highly stringent conditions. In Fig. 1, the genomic DNA isolated from rice Oc cells was digested with either *Bam*H I (lane 1), *Eco*R I (lane 2), *Xba* I (lane 3) or *Xho* I (lane 4) and hybridized with the radio-labeled probe derived from 5' end of the *OsPSK* cDNA. The result indicated that *OsPSK* is a single-copy gene. Indeed, screening of a genomic library allowed the isolation of only one group of genomic clones.

(Isolation of the *OsPSK* gene)

[0036] A genomic library was constructed with λEMBL3 phages and *Sau*3AI-digested DNA fragments prepared from rice Oc culture cells, and screened by plaque hybridization with the *OsPSK* cDNA as a probe. Three overlapping clones, namely, λEMBL3/3-1, λEMBL3/5-3, and λEMBL3/7-1,

carrying fragments derived from the *OsPSK* gene were obtained (Fig. 2B). These clones, classified into 3 subgroups, overlapped and spanned a region of more than 45 kp of the rice genomic DNA. To begin analyses of the *OsPSK* genomic structure and regulatory regions, the inventors carried out restriction mapping of the three λEMBL3 phage clones (Fig. 2B) by single and double restriction enzyme digestion as well as Southern hybridization. The resulting restriction map of the genomic fragment flanking the *OsPSK* gene is shown in Figure 2A. In Fig. 2, the restriction sites are abbreviated as follows : Ba;*Bam*HI, Bg;*Bgl*II, EI; *Eco*RI, EV; *Eco*RV, H;*Hin*dIII, P;*Pst*I. The 22.8-kb λEMBL3/7-1 insert was found to contain the entire *OsPSK* gene including the 5'-upstream region, the full-length transcribed sequence corresponding to *OsPSK* cDNA, the non-coding intron, and the 3'-downstream region. In Fig. 2C, the transcription direction of the *OsPSK* gene is marked by a horizontal arrow and the intron is shown by an open box. Noncoding and coding portions of *OsPSK* exons are indicated by shaded and striped boxes, respectively. The sequence encoding PSK-α within the second exon is depicted by a white bar. The horizontal bar labeled "327-bp" represents the probe used for genomic DNA hybridization.

(Determination of the transcription start site of the *OsPSK* gene)

[0037] The transcription start site of the *OsPSK* gene was determined by primer extension analysis and S1 mapping (Fig. 3). In Fig. 3, the reaction products of the S1 nuclease (lane 1) and the primer extension (lane 2) experiments were subjected to electrophoresis on a polyacryamide gel. The sizes of the probe used for S1 mapping and the products in both experiments are indicated in kb on the left. The inventors used one 32-base-long oligodeoxyribonucleotide corresponding to the 5'-end region of the *OsPSK* cDNA, as a primer (Fig. 4) and primer extension reaction was carried out. Total RNA extracted from Oc culture cells were used as a template, and products were analyzed by autoradiography after electrophoresis in a polyacrylamide gel. One band about 162-bases in

length was detected from the extension product (Fig. 3). Next, the inventors prepared a 315-bp probe using a 3.6-kb EcoRI fragment from the *OsPSK* gene and the ^{32}P-labeled primer followed by *Pst*I digestion. Then the inventors performed S1 nuclease analysis using the ^{32}P-labeled 315-bp fragment as a probe. After hybridization with total RNA and digestion with S1 nuclease, a product of 162-bases was again detected (Fig. 3). This size is identical to that from the 5'-terminal residue to the position complementary to the primer. Therefore, the first guanidine of the *OsPSK* cDNA, located 62 nucleotides downstream of the TATA box, was arbitrarily designated +1. Most likely it represents the 5' end of the *OsPSK* transcript (Fig. 4).

(Genomic organization of the *OsPSK* gene)

[0038] The nucleotide sequence of a 7.4-kb region harboring the entire *OsPSK* gene in the genomic clone λEMBL3/7-1 was completely determined on both strands and its structure analyzed. In Fig. 4, nucleic acid and deduced amino acid sequences of the *OsPSK* gene were shown. The transcription initiation site is designated +1 and a putative TATA-box is boxed. A CAAT-box, three E-boxes, three CCAAT-boxes, a enhancer core-like sequence, and three SSREs are indicated with underlining. The consensus binding site for the regulatory nuclear protein SEF3 is marked by a striped line up the sequence. The most likely sequence for a polyadenylation signal is labeled by double underlining. The sequence complementary to the primer used for primer extension and S1 nuclease analyses is indicated by a dashed line. The vertical arrows show the positions used in the subsequent construction of the various *OsPSK*-Intron-GUS fusion plasmids. The amino acid sequence is represented using the single-letter amino acid code. The amino acid sequence of PSK-α is indicated by the bold capital letters and the translation termination codon is marked with an asterisk. Sequence comparison of this fragment and the cDNA revealed that the *OsPSK* gene consisting of two exons (245 bp and 463 bp),

which perfectly match the cDNA sequence, is interrupted by a large intron of 1150 bp with a well-conserved GT-AG intron border sequence. An in-frame TGA stop codon was found 337 bp upstream of the Met codon of the first exon,including the 5' noncoding region and a coding region for about half of the PP-PSK (preprophytosulfokine), i.e. 48 out of 89 amino acid residues of the precursor, including the initiation methionine. A 22 amino acid NH_2-terminal hydrophobic region that presumably acts a signal was found in the first exon. The second exon was determined to consist of the 3' non-coding region and a coding region for the remaining 41 amino acid residues of the PP-PSK, within which the 5-amino acid PSK-α sequence occurred only once, close to the COOH-terminus. A poly adenylation signal consisting of an AAATTAA sequence was identified at positions 1629 to 1635.

(Characterization of the 5'-upstream region of the *OsPSK* gene)

[0039] The onventors searched the 5'-upstream region of the *OsPSK* gene for known motifs of other genes and found several potential regulatory elements (Fig. 4). The consensus sequence of a putative TATA box (5'-TATAA-3') was found at positions -63 to -68, referring to the transcription initiation site. Upstream to this sequence, there are one CAAT-box at -267 to -270 and three CCAAT-boxes at -906 to -910, -949 to -953, and -1074 to -1078, respectively. Interestingly, the sequence AACCCA (at -908) conforms to the A(A/C/G)CCCA consensus sequence, the binding site of a soybean enhancer for the regulatory nuclear protein SEF3 (Allen *et al.*, 1989), and an 8-nucleotide enhancer core-like motif (Weither *et al.*, 1983; Hata *et al.*, 1986), located at position -1105 to -1112 with the sequence 5'-GTGGAAAG-3'. Additionally, three E-boxes (consensus sequence: 5'-CANNTG-3'; Pabo, 1992), three shear-stress-responsive elements (SSRE: 5'-GAGACC-3'; Resnick *et al.*, 1993), and several repetitive sequences are present in this 5'-end region. These findings suggest that transcription may be influenced by a variety of genetic elements.

(Expression of *OsPSK*::GUS chimeric genes in transformed cells)

[0040] To determine the *OsPSK* 5'-upstream sequence required for its expression, the CaMV *35S* promoter in the plasmid pIG121 was replaced with various fragments from the 5' region of the *OsPSK* gene. In Fig. 5, the structure of *OsPSK*-Intron-GUS constructs were shown. A schematic diagram of the 5'-upstream region of *OsPSK* is shown at the top. The locations of the putative regulatory elements are indicated. Maps of constructs containing various portions of the 5'-upstream sequence (thick line) fused to the GUS gene (open box, not to scale) are shown next to their respective names. The indicated restriction enzyme sites are abbreviated as in Fig. 2. BSSEF3; the binding site of a soybean enhancer for the regulatory nuclear protein SEF3, ECS; enhancer core sequence, SSREs; shear-stress-responsive elements. Dashed lines indicate fragments in reverse orientation. As a positive control, the inventors used the original pIG121 containing the CaMV *35S* promoter (Ohta *et al.*, 1990). These constructs were introduced into Oc suspension culture cells via *Agrobacterium* infection, and transformed cells were selected on MS agar medium supplemented with 50 mg/l kanamycin (Yang *et al.*, 1999). For each construct, the inventors assayed GUS activity (Jefferson *et al.*, 1987) in more than 5 independent transformed cell lines. The results are presented in Fig. 6. The result of GUS activity is the mean of three independent experiments and indicated with the standard deviation.

[0041] The inventors constructed five *OsPSK*-Intron-GUS plasmids, pIG121-1, pIG121-2, pIG121-3, pIG121-4, pIG121-6, to determine the minimum length of the *OsPSK* 5'-upstream sequence required for maximal GUS activity. Transformed cells harboring these plasmids all displayed positive GUS activity and non-transformed cells as negative controls had no detectable GUS expression. The plasmid pIG121-1 harboring the shortest 5'-upstream region, which has no putative regulatory elements except TATA box, showed the lowest GUS activity.

The highest was recorded for the plasmid pIG121-4, within which most of the 5'-putative regulatory elements including SSRE and enhancer core motif are located, indicating that the region 1.9 kb upstream of the *OsPSK* transcription initiation site has the minimal amount of *OsPSK* sequence necessary for maximal GUS expression in transformed Oc cells. The cells transformed with pIG121-6, with the longest *OsPSK* 5'-upstream region, displayed similar GUS activity to cells transformed with pIG121-4.

[0042] To test for any potential enhancer-like activity within the *Bgl*II-*Hin*dIII fragment of the *OsPSK* 5'-upstream region in pIG121-4 but not in pIG121-3, the inventors constructed the plasmid pIG121-5 containing the *Bgl*II-*Hin*dIII fragment in the opposite orientation to that in pIG121-4. The GUS activity in cells transformed with this construct markedly decreased the activity recorded in cells transformed with pIG121-4 (Fig. 6). The inventors also constructed pIG121-7 in which the 2.3-kb *Eco*RI-*Hin*dIII fragment in pIG121-6 inserted in reverse orientation into pIG121-3. The plasmid pIG121-7 demonstrated GUS activity similar to that of the pIG121-3 and pIG121-5. These results suggested that the enhancer elements may be involved in the maximal GUS expression, with enhancement being orientation-dependent.

[0043] The GUS reporter gene under the control of the *OsPSK* 5'-upstream region yielded GUS activity approximately 2 to 5 times higher than that obtained with the GUS gene driven by the CaMV *35S* promoter in the transformed Oc cells, suggesting that it contains an efficient promoter for regulating the constitutive expression of a foreign gene in transformed rice Oc culture cells.

(Influence of exogenous auxin and cytokinin)

[0044] The inventors performed RNA blot analyses to investigate how exogenous phytohormones affect the expression of *OsPSK::GUS* gene in transformed Oc cells. Exogenous auxin (1 mg/l of 2,4-D) and/or cytokinin (1 mg/ l of 6-BA) were added into MS media to treat Oc cells transformed with

the plasmid pIG121-4. Total RNA was isolated from the various samples and subjected to Northern blot analysis. Exogenous auxin and cytokinin regulation of the *OsPSK* promoter is shown in Fig. 7. In Fig. 7, steady-state gusA mRNA levels in transformed Oc cells harboring pIG121-4 were examined by Northern blotting (left panel) and ribosomal RNA levels were examined by ethidium bromide staining (right panel). The transformed Oc cells were harvested 0 (lane 0), 12 (lane 1), 24 (lane 2), 48 (lane 3), and 72 h (lane 4) after auxin and/or cytokinin treatment. Total RNA was extracted from each sample and 20 μg was loaded into each lane. Fig. 7 shows the result of hybridization with the radio-labeled *Xba* I-*Sac* I fragment of pIG121 which contains the GUS coding region. Fig. 7A shows the result of MS medium supplemented with 2 mg/l 2,4-D, and the level of GUS mRNA increased significantly at 12 h after the onset of 2,4-D treatment, and reached a maximum level by 48 h (Fig. 7A). Fig. 7B shows the result of MS medium supplemented with 2 mg/l 6-BA and the expression of *OsPSK::GUS* gene was also reinforced by 6-BA 24 h after treatment, although decrease of GUS transcripts was apparent at 48 h (Figure 7B). Fig. 7C shows the result of MS medium supplemented with 1 mg/l 2,4-D and 1 mg/l 6-BA. The 2,4-D treatment combined with 6-BA resulted in a consistent increase in the mRNA level throughout the treatment period (Fig. 7C). These results suggested that both exogenous auxin and exogenous cytokinin may enhance the expression of *OsPSK* in Oc culture cells.

[0045] The inventors previously isolated *OsPSK* cDNA from rice Oc culture cells (Yang *et al.*, 1999). Gain-of-function and loss-of-function studies proved that *OsPSK* cDNA encodes the precursor of PSK-α, a peptide growth factor identified from plants. In the present investigation, the inventors identified and characterized the *OsPSK* gene in rice. Southern blotting analyses revealed *OsPSK* to be a single-copy gene (Fig. 1), consisting of two exons identical to the cDNA sequence, and a large intron (Fig. 2) conserved GT-AG intron border

sequence. The first exon contains the 5' noncoding region and a coding region for about half of the PP-PSK (preprophytosulfokine) including the 22 amino acid NH_2-terminal hydrophobic region that presumably acts a signal peptide (von Heijne, 1986). No signals that might cause retention along the secretory pathway (Nakai and Kanehisa, 1992) were detected in the sequence of PP-PSK, indicating that the protein may be extracellular and its active form, PSK-α, could therefore act as the ligand for its receptor. The second exon consists of the 3' non-coding region and a coding region within which the 5-amino acid PSK-α sequence occurs only once, close to the COOH-terminus.

[0046] Rice Oc cells (Baba *et al.*, 1986) can be simply maintained in/on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/l 2,4-D with dilution at regular intervals of 2 weeks. The inventors have previously shown that they can serve as an excellent starting material to study the synthesis and physiological function of PSK-α (Yang *et al.*, 1999; Yang *et al.*, 2000a). In the present study, the inventors constructed plasmids with the 5' upstream regions of the *OsPSK* gene fused to the Intron-GUS reporter gene (Ohta *et al.*, 1990) with a modified intron of the castor bean catalase gene within its N-terminal GUS coding sequence. When placed under the control of the CaMV *35S* promoter, the Intron-GUS reporter gene expresses GUS activity with a similar level and pattern as obtained with the original GUS reporter gene in tobacco cells but not in *Agrobacterium* cells (Ohta *et al.*, 1990). Furthermore, a significant stimulating effect on GUS synthesis by the intron has been observed in rice cells (Tanaka *et al.*, 1991), indicating that the Intron-GUS reporter gene is useful to monitor expression of foreign genes in rice cells. On fusing various lengths of the 5' upstream region of the *OsPSK* gene, the minimum length of the *OsPSK* 5'-upstream sequence required for the expression of maximal GUS activity was here found to be a region 1.9 kb upstream of the transcription start site (Fig. 3). This region is approximately 5 times more active than the CaMV *35S* promoter in

transformed Oc cells, suggesting that it contains an efficient promoter for regulating the constitutive expression of a foreign gene in transformed Oc culture cells.

[0047] Within the 5'-upstream region of the *OsPSK* gene, several known motifs or regulatory elements exist (Fig. 3). One of the most notable features of the *OsPSK* promoter is the presence of three CCAAT-boxes, which interact with CCAAT/enhancer-binding protein (Ryden and Beeemon, 1989). Furthermore, the inventors identified three SSREs, cis-acting components within the promoter of several human genes expressed in endothelial cells such as those for the platelet-derived growth factor β chain and transforming growth factor β 1 (Resnick *et al.*, 1993). They interact with DNA binding proteins and are necessary for fluid shear-stress responsiveness. Deletion of the region containing these regulatory elements resulted in rather low GUS expression (Fig. 6), suggesting that the SSREs may be actually involved in *OsPSK* expression. In addition, three E-boxes were found in the 5'-end region of *OsPSK*. The E-box, with a consensus sequence 5'-CANNTG-3', known as to be a recognition site for a class of transcription factors (basic region/helix-loop-helix proteins), can form homo- and hetero-dimers to exert regulatory function (Pabo, 1992). It is a variation of the G-box (5'-CACGTG-3'; Li and Capetanaki, 1994), elements of which comprise a family of cis-acting sequences that have been shown to be involved in the regulation of gene expression in response to variety of factors in plants as well as in animals (Baker *et al.*, 1994; Dolferus *et al.*, 1994). PSK-α promotes the growth and increase the chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions Yamakawa *et al.*, 1999), suggesting that the E-box conserved in the PSK-α precursor gene may have regulatory functions in the response to environmental stress.

[0048] Interestingly, an 8-nucleotide enhancer core-like motif, GTGGAAAG, exists in the 5'-upstream region of *OsPSK* (Fig. 3). This sequence is the most

common among known viral enhancers and dramatically increases the transcriptional activity of certain genes (Weiher *et al.*, 1983). It is also present in the 5'-upstream region of the human ornithine transcarbamylase gene (Hata *et al.*, 1987) and the available findings suggest that the enhancer sequence is universally conserved from viruses to eukaryocytes. The 1.9-kb 5'-upstream region of the *OsPSK* gene containing the enhancer core-like motif was here shown to be necessary and efficient for maximal-level GUS expression, and that this region was even more active than the CaMV *35S* promoter in transformed rice Oc cells (Fig. 5). The enhancer core-like motif in the *OsPSK* gene may have some functional role in the high-level expression of the *OsPSK::GUS* gene, this being dependent on the orientation.

[0049] When explanted into culture, many plant tissues dedifferentiate and resume division to form proliferating calli. Induction of cell division and subsequent callus formation normally require the simultaneous presence of both auxin and cytokinin. Hence, cell proliferation could be a direct response to auxin and cytokinin or an indirect response caused by PSK-α under their control. In suspension-cultured asparagus mesophyll cells, PSK-α can be produced only when both 1-naphthalene acetic acid and 6-BA are present in the medium. No significant amount of PSK-α is produced if either of these plant hormones is eliminated from the medium, suggesting that both auxin and cytokinin are normally required for the production of PSK-α (Matsubayashi *et al.*, 1999a). Here, the inventors could show that both exogenous auxin and exogenous cytokinin reinforce the expression of *OsPSK::GUS* in transformed Oc cells (Fig. 7). However, because exogenous hormones may not reflect the endogenous modulation of hormone levels, many more experiments remain to be done before drawing a final conclusion.

[0050] According to this invention, a novel promoter sequence derived from phytosulfokine precursor was provided. The potency of this promoter to

activate expression of a structural gene was higher than cauliflower mozaic virus *35S* promoter.

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